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International Bureau

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(21) International Application Number: PCT/SE89/00539 (22) International Filing Date: 2 October 1989 (02.10.89) (30) Priority data: 8803496-2 3 October 1988 (03.10.88) SE (71)(72) Applicant and Inventor: NILSSON, Kurt, G., I. [SE/ SE]; Andjaktsv. 6, S-222 53 Lund (SE). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (Euro- pean patent), JP, LU (European patent), NL (European patent), SE (European patent), US.		Published <i>With international search report.</i> <i>With amended claims.</i> <i>In English translation (filed in Swedish).</i>
(54) Title: NANOPARTICLE WITH A BOUND ENZYME AND ANOTHER LIGAND USEFUL IN ANALYSIS (57) Abstract The invention relates to a reagent consisting of at least one enzyme and at least one other substance, which are covalently or noncovalently bound to a particle, which is smaller than or is equal to 1000 Angstrom in diameter. The invention also relates to method and use of the reagent for determination or studies of a cell or a virus or another component in a sample. According to the invention, these determinations are made with for example some ELISA-technique (competitive, sandwich, etc), employing the reagent preferably in the form of a suspension in buffered water, instead of and in the same way as described for soluble enzyme conjugates. The substance can be an antibody, a lectin, avidin or an antigen, the enzyme can be for example peroxidase, alkaline phosphatase, and the particle can be an inorganic or an organic polymeric compound or a combination thereof, as, for example, tresyl-activated glycerylpropylsilica.		

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NANOPARTICLE WITH A BOUND ENZYME AND ANOTHER LIGAND USEFUL IN ANALYSIS

The present invention relates to a new type of reagent to be used in the determination of a cell, a virus or another component of a test and to the use of the reagent in diagnostical, clinical, histochemical or microscopical applications.

BACKGROUND

Chemical analysis based on specific biological affinity between receptor and ligand, as for example between antibody and antigen, between lectin and carbohydrate, between nucleotides etc. has been used for several years and their use in the separation and determination of cells, virus or other components in samples has increased lately. Examples of this are analysis based on antibodies, i.e. the immunotechniques. Antibodies are here used for the specific binding of the analyte in the sample. Detection of antibody-ligand complex or of free antibody can be done in several ways. In the so called radioimmunoassay, RIA, radioactively marked antibodies or ligands are used, but also for example bacteriophages, free radicals, fluorescent or luminiscent groups, different enzymes and particles have been used as markers in immunoassays (see e.g. Ngo and Lenhoff, editors, Enzyme-mediated immunoassay, pages 3-32, Plenum Press, 1985, ref. 1). RIA has however dominated. In spite of the advantages of RIA, disadvantages as for example the instability of several gamma-emitters and the health hazards by the synthesis and the handling of the radioactive ligands or antibodies have lead to the gradual replacement of the radioactive markers by other types of markers. Especially enzyme immunoassay (EIA; ref. 1 and Engvall,

Meth. Enzymol., 70, pages 419-439; ref. 2) has turned out to have advantages and is more and more used. EIA is divided into homogeneous and heterogeneous EIA. Heterogeneous EIA, which includes ELISA (enzyme linked immunosorbent assay) is based on the same principles as RIA and very often gives similar sensitivity and specificity. These immunotechniques and techniques based on other types of biospecific interactions for analysis of cells, virus or other components in samples, require methods for the conjugation of receptor or ligand to any type of marker substance (e.g. an enzyme or a radioactive substance). Even if several such methods based on chemical reagents are described in the literature (for example glutaraldehyde, periodate or thiol substances; see O'Sullivan and Marks Meth. Enzymol. vol. 73, pages 147-166, ref. 3, ref. 2 and other relevant articles in Meth. Enzymol., volumes 70, 73 and 92), they all have their drawbacks. It is for instance difficult with existing techniques to produce, reproducible and controllable, for example enzyme-antigen, enzyme-antibody or enzyme-lectin conjugates and other types of enzyme-ligand or enzyme-receptor conjugates. One obtains conjugates which are heterogeneous in size and structure. Furthermore, conjugation may cause decreased enzyme activity or specificity of the ligand or receptor. Furthermore, even if the sensitivity often is sufficient, EIA is in many cases limited by insufficient sensitivity. One has tried to increase the sensitivity by using more or less complicated systems as avidin-biotin (Kendall et al, J. Immunol. Meth., vol. 56, page 329, 1983, ref. 4, see also ref. 1-3 and relevant articles in Meth. Enzymol.) or so called enzyme-cascades (Self, US Patent Application No. 307, 600; ref. 5).

A more simple system utilises soluble, oligomeric enzyme coupled to antibody. Leary et al, Proc. Nat. Acad. Sci., vol 80, page 4045, 1983; ref.6). Even if these so called amplification techniques are useful in several applications, they have their limitations and disadvantages and Ngo mentions in a review that increased sensitivity of EIA and better methods for the preparation of enzyme-ligand or enzyme-receptor conjugates are desirable.

One of the objects of this invention is to reduce these disadvantages by EIA and a simple method to form enzyme-antigen, enzyme-antibody or enzyme-lectin conjugates, as well as conjugates between enzyme and other types of ligands or receptors, are described. Furthermore, it is described how this reagent can be used for the detection of a cell, a virus or another component in a sample and how an increased sensitivity compared to earlier methods can be reached by using the reagent according to the invention.

These and other aims can be reached, according to the invention, by covalent or noncovalent binding of enzyme and antibody or enzyme and lectin or enzyme and antigen (or in general, enzyme and ligand or receptor) to suitable type of particles, which are insoluble in the medium (usually buffered water) used in the conjugation reaction to the particle and in the determination of the analyte, and, furthermore, by using thus formed reagent, instead of and in the same way as previously described enzyme conjugates, for the specific detection of a cell, a virus or another component in a test.

By changing the size of the particle, the chemical and physical structure, type of surface (hydrophobic, hydrophilic, with or without reactive groups), enzyme, antibody or antigen (another type of receptor or ligand) and, furthermore, by changing their

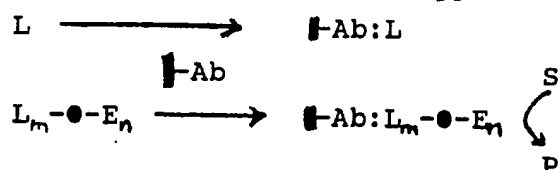
concentrations, one can obtain particle conjugates with, for the application, suitable properties.

Examples of applications of the reagent according to the invention are in ELISA (ref. 1 and 2), in immunohistochemical studies (Avrameas, Histochem. J. vol. 4, page 321, 1972; ref. 7) immunoblotting (Tsang et al, Meth. Enzymol. vol. 92, page 377; ref. 8) in microscopical studies, for the detection of antibodies in cell cultures etc.

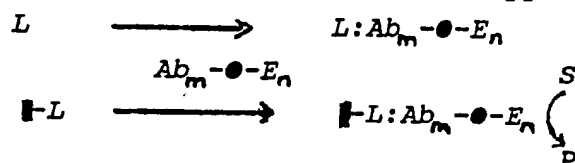
As examples of techniques where the reagent according to the invention, can be used, are competitive and noncompetitive ELISA-methods and in USERIA (Meth. Enzymol. vol. 73, page 383 Hsu et al; ref. 9). The soluble enzyme conjugates that have been used earlier in these methods are consequently replaced with the reagents according to the present invention. Otherwise, the techniques when carrying out the assays are identical or similar with the in literature described EIA-techniques (ref. 1.2 etc.). Some examples of ELISA principles with which the reagent according to the invention can be used is shown in the annexed schemes (page 5, where L is the analyte, that is the component which will be determined, Ab is antibody or lectin, carbohydrate, coenzyme etc, with specific affinity to L, E is enzyme, S is enzyme substrate and P is product, which is detected, $L_m-\bullet-E_n$, and $Ab_m-\bullet-E_n$ symbolise examples of reagents according to the invention, $-\bullet-$ is the particle and \blacksquare is the solid phase).

In references 1 and 2 given above (and references given in these articles) the ELISA-techniques with soluble enzyme conjugates are explained extensively, as well as the buffers and other reagents involved and the applications, and the techniques and the applications when using the reagent according to the invention are similar or identical.

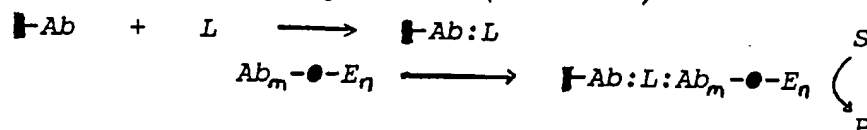
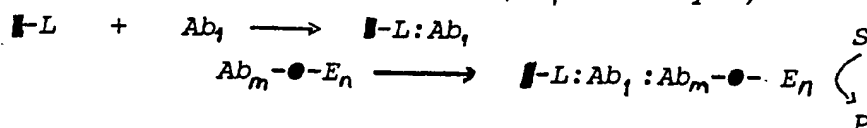
Competitive type of ELISA



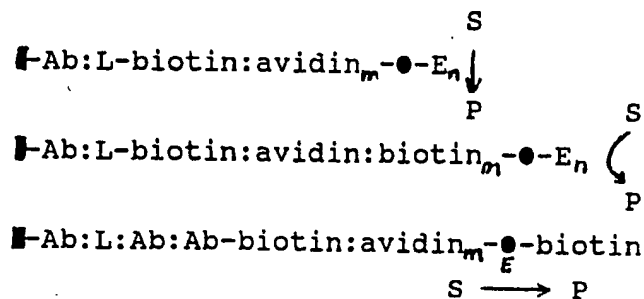
Inhibition type



Noncompetitive (sandwich)

Indirect (Ab_1 = analyte)

The reagent according to the invention can also be used together with many of the amplification techniques described for ELISA (avidin-biotin, enzyme-cascade, USERIA etc.). Thus, as an example, with avidin-biotin, avidin (A) and enzyme (E) can be coupled to the particles (●) and the resulting reagent can be used for the detection of bound biotin-labelled antibody or analyte and, for example, the final complexes shown in the annexed scheme can be detected.



(etc; Dakopatts Product list, 1988; ref. 10).

For review articles on avidin-biotin, see HSU et al page 467 in Ngo and Lenhoff (ref. 1) and Wilchek and Bayer, Immunology Today, vol. 5, page 39, 1984 (ref. 11). The sensitivity can be increased, by further reacting the product P, formed in the above enzyme reactions with other enzymes (according to for example ref. 5 above) or by transforming P to a luminiscent substance according to for example Wannlund and Deluca, Meth. Enzymol. vol. 92, page 426 (ref. 12).

The final product is preferably detected with conventional ELISA-technique, that is by the eye, by measuring the absorbance, fluorescens, etc. or the reaction is followed kinetically (Tsang et al, Meth. Enzymol. vol. 92, page 391; ref. 13). Furthermore, the product P can be radioactive and can be separated from the substrate with for example a Sephadex-column and be quantified with a scintillation counter (USERIA; ref. 9 above). The above constitutes only examples of how the invention may be used in actual practice and are not intended to limit the scope of the invention. The same is valid for the following examples of particle-type, conjugation chemistry, enzymes, ligands or receptors, type of samples and components and types of solid phases that can be used in the application of the invention.

The particles can consist of a polymer substance which is soluble in water and can consist of natural, semisynthetic or synthetic materials. As examples one can mention silicates, borosilicates, zeolites, aluminates, inorganic particles, the surface of which have been modified with organic materials containing for example alkyl groups, aromatic groups, hydroxyl groups, epoxi groups, aldehyde groups, esters, plastics (polyvinylalcohol, polystyrene, etc.), copolymers (e.g. Eupergit and Dynospheres) cross-linked polysaccharides, liposomes, artificial cells, etc.

Immobilization of the enzyme and ligand or receptor to the particles can easily be carried out by a person skilled in the art and does not limit the scope of the invention. The surface of the particles can be more or less hydrophobic. A hydrophobic surface can be used for noncovalent binding of enzyme and ligand or receptor to the particle. Both hydrophobic (for example polystyrene particles, which can be nitrated, reduced and diazotized) and hydrophilic surfaces can be chemically modified to introduce reactive groups for covalent binding and the literature is extensive in this field. (See for instance Methods Enzymology volumes 44, 104 and 135.)

For example, diazo, cyanate, ester, tosyl or tresyl groups, aldehyde, epoxi, divinylsulphone, FMP-groups can easily be prepared. After this so called activation step, the so called coupling of the enzyme and the ligand, simultaneously or consecutively, to the particle is carried out. After the coupling, performed at suitable pH and temperature, the particles are washed after centrifugation and the reagent can then be used in, for example, those types of EIA mentioned above. The concentration of the particles in the coupling step is kept at a suitable level to avoid crosslinked particles. The so prepared particles with bound enzyme and ligand or receptor, can eventually be size separated using e.g a Sephadex-column. The ratios ligand/enzyme or receptor/enzyme are chosen after the application. A high ratio of enzyme to receptor or ligand can give a high enzyme activity but can result in a low binding capacity. A person skilled in the art can easily decide the optimal conditions for a given situation. Eventually the enzyme can be coupled first and then the ligand or the receptor can be coupled either directly on the remaining particle surface or on the enzyme with the help of crosslinker

of the type glutaraldehyde or with the help of a reagent of the type periodate. One can also according to the invention, couple a conjugate between enzyme and ligand or enzyme and receptor to the particles. One obtains an increased sensitivity with the reagent according to the invention compared to the corresponding soluble conjugate because of the multiple enzyme molecules per particle and per component in the sample.

The size of the particles are chosen with regard to the application. Preferably particles with the diameter of < 500 Angstroms are used, to minimize the risk that the reagent according to the invention, is washed off after the binding step in e.g. a sandwich-ELISA. This size allows more enzyme molecules and receptors or ligand molecules to bind to each particle and also allows a homogeneous particle suspension. Examples of commercially available particles of suitable size are Degussas silica particles Aerosil. The hydrophilic Aerosilparticles can easily be silanized and activated with, for example, tresyl clorid (Nilsson and Mosbach, Meth. Enzymol. vol. 104, page 56; ref. 14) periodat, etc.

The choice of enzyme and enzyme substrate can easily be done by a person skilled in the art and does not limit the scope of the invention. All enzymes, which can be coupled to particles, can be used according to the invention. Any enzyme and substrate, which are suitable for the application can be used, and the same enzymes and substrates which have been used in ELISA, immunohistochemical studies, immunoblotting and microscopical studies can be used. Examples of enzymes which can be used are peroxidase, alkaline phosphatase, galactosidase, urease, glucose-6-phosphate dehydrogenase and luciferase. To increase enzyme activity per particle an enzyme with high turnover

number and relatively low molecular weight can be used. Examples of enzyme substrates which can be used together with the reagent according to the invention, are given in references 1-13 and in relevant articles in Meth. Enzymol. volumes 70, 73 and 92.

As mentioned above, the ligand or the receptor, that is the substance which is coupled to the particle together with the enzyme, shall be able to biospecifically bind either the analyte (that is the component which is to be detected in the sample) or an antibody, another protein (lectin, avidin etc.) a coenzyme, carbohydrate etc. The substance, which is coupled to the particle together with the enzyme, can be identical with the analyte or be an analogue thereof. This substance can for instance be an antibody, a lectin, avidin, another sort of protein or glycoprotein, a carbohydrate derivative, a glycolipid, a neoglycoprotein, a steroid derivative, a coenzyme derivative, a metabolite derivative, an analogue or metabolite of a pharmaceutical preparation, a metabolite, hormone, nucleotide or a derivative thereof, another virus or cell component or a derivative thereof.

The sample, which contains the cell, virus or component, can be in the form of a fluid (tears, saliva, serum, urine, water sample, etc.) in the form of more or less solid material (tissue, nitrocellulose etc.). The cells, virus or components which can be analyzed with the reagent according to the invention, are for example pathogenic organisms such as parasites, yeast cells, bacteria, mycoplasma, virus, toxins, pharmaceutical preparations and their metabolites, other metabolites, hormones, antibodies and other proteins, steroids, prostaglandins, carbohydrates, glycoconjugates, nucleotides and biomolecules in cells, virus, on cell surfaces, in tissues or in the

circulation and other cells, virus or components for instance in waste water, earth, plants, animals and food.

The solid phase used for the separation step in ELISA with the reagent according to the invention, can be of the same type that have been used in ELISA with earlier described enzyme conjugates (see e.g. pages 388-391 in Meth. Enzymol. vol. 70, 1980), plastics in the form of test-tubes, microtiter plates, particles, filters, etc., glassfiber or filters of paper, ion exchangers, agarose particles, Sephadex particles, polyacrylamid gel, bentonite, magnetic preparations of cellulose, agarose or plastics, etc. The choosed shape of the solid phase depends on the application and for instance balls, columns, dipsticks, microtiterplates, membranes, filters or test-tubes can be used.

After the separation of the bound enzyme particles from the non-bound, the activity of either the bound fraction or of the non-bound fraction is determined.

Some examples of how the invention may be used in actual practice are described in the following Examples, which in no way is intended to restrict the scope of the invention.

EXAMPLE

Activation of silica particles.

Silica particles from Degussa (400 mg, Aerosil TT 600) was silanized in a vacuumexiccator with γ -glycidoxypropytrimetoxysilane (300 microlitre) using trimethylamine (300 microlitre) as catalyst at 140°C for 4 hours. The procedure was repeated. The epoxi groups was hydrolysed with water and HCl (pH 3) at 90°C for 30 minutes. Centrifugation, wash with water and

gradual transfer to acetone according to ref. 14, followed by activation of the hydroxyl groups with tresyl chlorid (30 microlitres per 150 mg particles) in 1 ml acetone and with 40 microlitres pyridine at 0°C for 20 minutes, wash according to ref. 14, (with centrifugation of the particles), gave particles with 150 micromole tresyl groups per g particles according to elementary analysis.

Coupling of peroxidase and rabbit anti-human transferrin immunoglobulin and of peroxidase and rabbit anti-human α -1-fetoprotein immunoglobulin to tresyl-activated Aerosilparticles.

Peroxidas (12 mg, purified with affinity chromatography, Sigma) and antitransferrin (1.5 ml, dialysed with 0.3 M sodium-bicarbonate, pH 8.5; Dakopatts), were slowly mixed during stirring with tresyl activated silica particles (250 mg wet-weight in 4 ml buffer) and ultrasoundtreated for 3 minutes. Coupling proceeded with agitation on an end-over-end table for 70 hours at 4°C. The particles were washed with coupling buffer (4 times) using centrifugation of the particles. Bovine serum albumin (40 mg in 5 ml) was added to block any eventually remaining tresyl groups and the particles are stored at 4°C. Spectrophotometric determination of the absorbance of the wash-solutions at 403 nm and 280 nm indicated that 2 mg peroxidase and 2 mg antitransferrin had been coupled. Peroxidase (15 mg, 1000 U/mg, Boehringer, ELISA-quality) and anti- α -1-fetoprotein immunoglobulin (50 mg, Dakopatts) were coupled at 4 °C for 40 hours in almost the same way but with 8.5 ml coupling-buffer (see above) and by the gradual addition of tresyl-activated silica particles (25 mg dry weight in 1 ml buffer). Remaining tresyl

groups were blocked with Tris-HCl, pH 8.3, 0.2 M.

Determination of human transferrin with sandwich-ELISA using peroxidase-antitransferrin-silica:

Step 1. A microtiterplate was incubated with 300 microlitre in each well of 10 microgram rabbit antitransferrin immunoglobulin (Dakopatts, AO 61, lot 012) dissolved in PBS (10 mM sodium phosphate + 145 mM NaCl, pH 7.2). Incubation at 4 °C over night. The plate was washed with PBS containing 0.1 % Tween 20 and 0.5 NaCl (buffer B).

Step 2. The plate was incubated with a dilution serie (10 microgram to 0.02 ng) of human transferrin (apoform, Sigma) dissolved in buffer B. Incubation for 2 hours at 20 °C.

Step 3. Peroxidase-antitransferrin-particles prepared as above were suspended in buffer B and added to the wells. Incubation for 3 hours at 20 °C with gentle agitation on a shaker. The plate was washed 3 times with buffer B and substrate solution (150 microlitre of 8 mg 1,2-phenylenediamine dihydrochloride dissolved in 0.1 M citric acid/phosphate buffer pH 5.0, 12 ml, and 12 microlitre H₂O₂) was added to the wells (step 4).

After 50 minutes the absorbance of the wells was measured with a multiscanner at 492 nm. The wells which in step 2 were incubated with less than 2 ng transferrin gave higher absorbance than the background absorbance (obtained in wells which in step 2 were incubated without antigen, that is, with only buffer B).

Determination of human α -1-fetoprotein with sandwich-ELISA using peroxidase-anti- α -1-fetoprotein-silica:

Human α -1-fetoprotein (Dakopatts, X 900 standard, lot 014) was determined with same procedure and buffers used in the above example but with the difference that silica particles with co-immobilised peroxidase and anti- α -1-fetoprotein immunoglobulin (prepared as described above) were used. In step 1 the wells were incubated with anti- α -1-fetoprotein immunoglobulin (rabbit, Dakopatts, A008, lot 085) and in step 2 α -1-fetoprotein was added to the wells in a dilution serie from 1 microgram to 0.1 ng. In step 3 soluble conjugate of peroxidase and antihuman α -1-fetoprotein was used (rabbit, Dakopatts, P 128, lot 046) for comparison with the silica conjugate. Wells, that in step 2 were incubated with 0.1 ng antigen and in step 3 incubated with enzyme-antibody-particles gave absorbance which was more than twice as high as the background absorbance (obtained in wells where no antigen was incubated in step 2). The soluble enzyme-antibody conjugate gave no absorbance difference for 0.1 ng antigen. Both soluble and particle bound conjugate gave ca 10 times higher absorbance in wells which in step 2 were incubated with 1 microgram antigen compared to the absorbance obtained in the blank wells.

CLAIMS

1. A reagent, characterised by that at least one enzyme and at least one other substance is covalently or noncovalently bound to a particle which is smaller or which is equal to 1000 Angstroms in diameter.
2. Method and use of a reagent as claimed in claim 1, for the determination of a cell, a virus or a component thereof or another type of component in a sample with ELISA-technique and similar enzymatic techniques, for immunohistochemical studies, for immunoblotting, for microscopical studies, etc, in which techniques or studies the reagent according to claim 1 is used in the same way and instead of previously described enzyme conjugates, i.e. for the specific binding and for the catalytic transformation of enzyme substrate to detectable product or to product which can be detected after transformation via for example an enzyme cascade.
3. Other method or use of reagent as claimed in claim 1.
4. Reagent as claimed in claim 1-3, characterised by that the particle consists of a polymer or a derivative thereof, which have a low or no solubility in water under the conditions which are employed at the method or use of the reagent.
5. Reagent as claimed in claim 1-4, characterised by that the particle consist of a natural, semi-synthetic or synthetic material as for example a polysaccharide, glass, silicate, borosilicate, aluminate, zeolite, crosslinked or

surface-modified organic or inorganic polymer, plastic, copolymer, or an artificial cell.

6. Reagent as claimed in claim 1-5, characterised by that the surface of the particle has hydrophobic or hydrophilic groups or has reactive groups as some of diazo, epoxi, aldehyde, cyanate ester, tosyl, tresyl, an other ester, acyl azide, aromatic carbamate, triazine, succinimide, carbodiimide, imidate, disulphide, reactive halogen, divinylsulphone, FMP, or a photoactive substance, etc., for covalent coupling of enzyme and substance to the particle.

7. Reagent as claimed in claim 1-6, characterised by that an enzyme and a substance have been covalently bound, employing reactive groups as tresyl groups, to a silica particle, the surface of which has first been modified with - glycidoxytrimethoxysilane, then hydrolysed at weakly acidic pH and thereafter been activated with for example tresyl chloride.

8. Reagent as claimed in claim 1-6, characterised by that the enzyme and the substance are bound simultaneously or after each other to the particle or that a conjugate between the enzyme and the substance is first formed and which is then coupled to the particle or that the enzyme and the substance are crosslinked after adsorption to the particle surface with a crosslinker like for example glutaraldehyde.

9. Reagent as claimed in claim 1-8, characterised by that the enzyme is one of for example alkaline phosphatase, urease,

-galactosidase, lysozyme, proteases, other hydrolase, glucose oxidase, peroxidase, glucose-6-phosphate dehydrogenase, other oxidoreductase.

10. Reagent as claimed in claim 1-9, characterised by that the substance is for example an antibody, a lectin, avidin, other protein, polysaccharide, carbohydrate or a derivative thereof, steroid derivative, prostaglandin derivative, hormone or a derivative thereof, pharmaceutical or a metabolite or an analogue thereof, nucleotide, nucleic acid or a derivative thereof, other cell or virus component.

11. Use of a reagent claimed in claim 1 for immunological determinations in for example a diagnostic kit.

AMENDED CLAIMS

[received by the International Bureau
on 1 March 1990 (01.03.90);
original claims 1-11 replaced by amended claims
1-8 (3 pages)]

1. A reagent, characterised by that at least one enzyme and at least one other substance are covalently or noncovalently bound to a particle, which has a low or no solubility in water under the conditions employed at the method or use of the reagent, and which is smaller or equal to 1000 Å (A.U.) in diameter.
2. Reagent as claimed in claim 1, characterised by that the particle consists of a natural, semi-synthetic or synthetic material as for example a polysaccharide, glass, gold, silicate, borosilicate, aluminate, zeolite, crosslinked or surface-modified organic or inorganic polymer, plastic, copolymer, or an artificial cell.
3. Reagent as claimed in claim 1-2, characterised by that the surface of the particle has hydrophobic or hydrophilic groups or has reactive groups as one of diazo, epoxi, aldehyde, cyanate ester, tosyl, tresyl, an other ester, acyl azide, aromatic carbamate, triazine, succinimide, carbodiimide, imidate, disulphide, reactive halogen, divinylsulphone, FMP, or a photoactive substance, etc., for covalent coupling of enzyme and substance to the particle.
4. Reagent as claimed in claim 1-3, characterised by that an enzyme and a substance have been covalently bound, employing reactive groups as tresyl groups, to a silica particle, the surface of which has first been modified with γ -glycidoxypropyltrimethoxysilane, then hydrolysed at

weakly acidic pH and thereafter reacted with for example tresyl chloride.

5. Reagent as claimed in claim 1-4, characterised by that the enzyme and the substance are bound simultaneously or after each other to the particle, or that a conjugate between the enzyme and the substance is first formed and which thereafter is coupled to the particle, or that the enzyme and the substance are crosslinked after adsorption to the particle surface with a crosslinker like for example glutaraldehyde.

6. Reagent as claimed in claim 1-5, characterised by that the enzyme is one of for example alkaline phosphatase, urease, galactosidase, lysozyme, proteases, other hydrolase, glucose oxidase, peroxidase, glucose-6-phosphate dehydrogenase, other oxidoreductase.

7. Reagent as claimed in claim 1-6, characterised by that the substance is for example an antibody, a lectin, avidin, other protein, polysaccharide, carbohydrate or a derivative thereof, steroid derivative, prostaglandin derivative, hormone or a derivative thereof, pharmaceutical or a metabolite or an analogue thereof, nucleotide, nucleic acid or a derivative thereof, other cell or virus component.

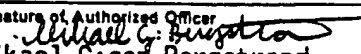
8. Method and use of a reagent as claimed in claim 1, for the determination of a cell, a virus or a component thereof or another type of component in a sample with ELISA-technique and similar enzymatic techniques, for immunohistochemical studies, for immunoblotting, for microscopical studies, etc, in which techniques or

studies the reagent according to claim 1 is used in the same way and instead of previously described enzyme conjugates, i.e. for the specific binding and for the catalytic transformation of enzyme substrate to detectable product or to product which can be detected after transformation via for example an enzyme cascade.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 89/00539

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: G 01 N 33/544, C 12 N 11/02, C 12 Q 1/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ?		
Classification System :	Classification Symbols	
IPC5	G 01 N; C 12 N C 12 Q	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT*		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP, A2, 0155224 (CHROMAGENICS, INC.) 18 September 1985, see the whole document	1-6,8- 11
Y	--	1-6,8- 11
X	EP, A1, 0077671 (ORTHO DIAGNOSTIC SYSTEMS INC.) 27 April 1983, see the figures, page 6 lines 15-36 page 9 line 19 - page 11 line 4	1-6,8- 11
Y	--	1-6,8- 11
Y	US, A, 4267235 (REMBaum ET AL) 12 May 1981, see especially the abstract	1-6,8- 11

<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁴</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
30th January 1990	1990 -01- 3 1	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	 Mikael G. son Bergstrand	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers 3 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The wordings "Other methods or uses" are too broadly formulated to permit a meaningful search. The search on claim 3 has therefore been incomplete.

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 89/00539**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0155224	18/09/85	JP-A- 60218071	31/10/85
EP-A1- 0077671	27/04/83	JP-A- 58079162	12/05/83
		US-A- 4434150	28/02/84
		CA-A- 1200760	18/02/86
US-A- 4267235	12/05/81	NONE	